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Structure of Pseudocerosine, an Indolic Azafulvene Alkaloid from the Flatworm *Pseudoceros indicus*

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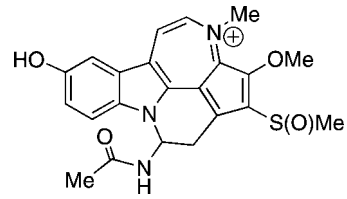
ABSTRACT



The rim of the tunic of the flatworm *Pseudoceros indicus* is characterized by blue dots on a white background. The isolation and structure elucidation of the blue pigment is reported. It is shown by extensive analysis of spectroscopic data to be an indolic azafulvene, which has been named pseudocerosine.

Marine biota continue to provide unique structures with compelling biological activity and/or structural interest. As part of our bioprospecting effort aimed at the discovery of new leads for antitumor compounds, we had the opportunity to sample the marine flatworm *Pseudocerus indicus*, which was collected by snorkeling in mangrove forests of Palau, Chuuk, and Pohnapei (Micronesia).[1] The animal was observed in high abundance in between the mangrove roots, often feeding on the white colonial ascidian *Eudistoma toealensis*. We previously have described a series of staurosporine-type indolocarbazole alkaloids from the flatworm and its ascidian prey.[2−4] In this paper, we describe the structure elucidation of the blue pigment, which forms the characteristic, well-defined blue spots along the body margin of the otherwise off-white mantle.[5] Compound **1** was isolated as an intensely blue colored microcrystalline solid in 0.025% yield (dry weight). Optical activity could not be established because of the intense color of the compound (λ_{max} (log ε) 218 (4.31), 260 (4.33), 310 (4.36), 405 (3.40), 569 (3.83), 602 (3.86) nm). The ESI-TOFMS spectrum displayed a molecular ion at *m*/*z* 424.1326 indicative of a molecular formula of $C_{22}H_{22}N_3O_4S$ (−0.5 mmu error). This putative molecular formula was corroborated by the presence of 22 distinct signals in the ^{13}C NMR spectrum of **1** between 22.7 and 172.6 ppm (see Table 1). Most signals in both the proton and the ^{13}C domain displayed doubling, with a ratio of major to minor of approximately 5:2. On the basis of extensive NMR data analysis, the structure shown in Figure 1 was assigned to compound **1**, which we named pseudocerosine.

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(5) These spots distinguish *P. indicus* from *P. glambei*, which has a continuous purple rim. Pseudocerosine is the pigment contained in these spots, the color of which is reflected in the species name *P. indicus* (from the Latin *indicus* = blue ink).

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Table 1. NMR Data (500 MHz) for **1**[a] in MeOH-d_4

C/H no.	δ_C[b]	δ_H (mult., J in Hz)	HMBC[c]	NOE[d]
1	152.8	8.22, d (6.8)	H-2, H_3-20	H_3-20
2	106.9	7.32, d (6.8)	H-1	
3	148.6		H-1, H-5, H-8[e]	
4	119.5		H-2, H-8	
5	109.9	7.45, d (2.6)	H-7	
6	155.9		H-5, H-7, H-8[e]	
7	127.6	7.33, dd (9.3/2.6)	H-5	
8	118.5	7.52, d (9.3)		H-17
9	132.6		H-5, H-7	
10	119.7		H-2	
11	140.2		H-16a, H-16b, H-17	
12	121.0		H-16a, H-16b, H-17	
13	144.6		H-16a, H-16b,[e] H_3-22	
14	137.0		H-16a,[e] H-16b,[e] H_3-21	
15	132.3		H-1, H-16a,[e] H-16b,[e] H_3-20	
16a	35.8	4.18, dd (18.3/9.5)	H-17[e]	H_3-22
16b		3.93, dd (18.3/2.5)		
17	69.3	6.92, dd (9.5, 2.5)	H-16b, H_3-19[e]	H-8
18	172.6		H-17, H_3-19	
19	22.7	1.94, s		
20	46.4	4.28, s	H-1	H-1, H_3-21
21	66.4	3.93, s		H_3-20, H_3-22
22	42.2	3.13, s		H-16a, H_3-21
NH		7.35, d (8.6)[f]		

[a] Data for major diastereomer. [b] Recorded at 125 MHz. [c] HMBC optimized for $J = 10$ Hz. [d] Important 1D-NOE interactions. [e] HMBC optimized for $J = 3$ Hz. [f] ^{1}H NMR data in MeOH-d_3.

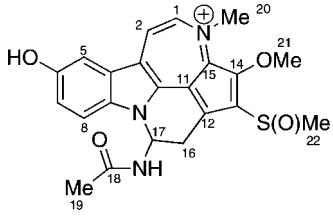

Figure 1. Structure of pseudocerosine (**1**).

Interpretation of gHSQC data suggested that the 22 carbon atoms were present in the form of four methyl, one methylene, six methine, and 11 quaternary carbon atoms, which meant that two exchangeable protons were present. On the basis of gCOSY data recorded in MeOH-d_4 and MeOH-d_3, respectively, three spin systems were readily assembled as shown in Figure 2. For fragment **a**, the coupling pattern of H-5, H-7 and H-8 suggested that they were located in a 1,2,4-trisubstituted aromatic ring.

Furthermore, the deshielding evident in the chemical shifts of H-5/H-7 and of H-8 in conjunction with the ^{13}C chemical shifts of the attached carbon atoms (109.9, 127.6, 118.5 ppm) was suggestive of an indole system.

Another two-proton spin system consisted of a vinyl group resonating at 8.22 ppm and 7.32 ppm, respectively, and the J-coupling suggested that these two protons were located ortho to each other (fragment **b** Figure 2). A third spin system revealed by the gCOSY spectrum consisted of three signals in the form of an ABX system (fragment **c** Figure 2), which showed an additional coupling to the X-spin when the spectrum was recorded in MeOH-d_3. Fragments **a** and **b** were linked on the basis of gHMBC correlations to the two quaternary carbon atoms resonating at 148.6 and 119.5 ppm, respectively. The partial structure was further expanded to include a quaternary N-methyl group (δ_H 4.28; δ_C 46.4) at the other end of the two-spin system yielding substructure **e** (Figure 3).

Fragment **d** (Figure 3) was established on the basis of gHMBC data and was linked to fragment **c** on the basis of ^{15}N-gHMBC and 1D-NOE data. Specifically, H-16b showed ^{15}N-correlations to two nitrogen atoms resonating at δ_N −236 and −249, respectively (Figure 3 and Figure S-4 in the Supporting Information). The latter ^{15}N resonance also correlated to the acetate methyl whereas the former correlated to H-8 of fragment **a**. This suggested the presence of an aminal function within **1** in which the aminal proton was close to H-8 of fragment **a** as evidenced by a NOE correlation

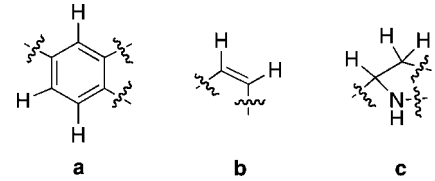

Figure 2. Fragments assembled from gCOSY data.

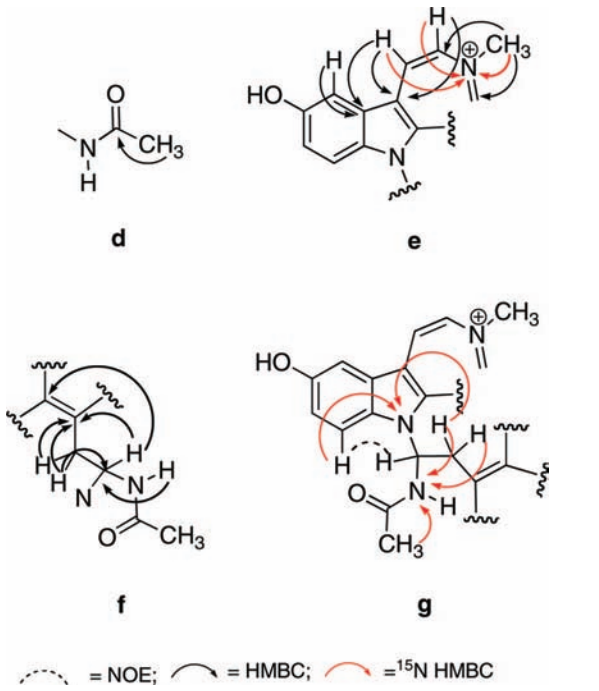

Figure 3. Substructures assembled on the basis of H,C- and H,N-HMBC.

between them. Analysis of gHMBC spectra optimized for small and large coupling constants, respectively, suggested that C-16 was attached to a quaternary olefinic carbon atom resonating at 121.0 ppm. This carbon atom was bonded to two other quaternary olefinic carbon atoms, resonating at δ_C 140.2 (C-11) and δ_C 144.6 (C-13), respectively, as shown by ^3J and ^4J HMBC correlations to both H-16a/b and H-17 for the former and ^3J correlations to H-16a/b for the latter.

At this point in the analysis, all of the carbon and hydrogen atoms had been accounted for. The remainder was "proton-poor" and had to be assembled without the benefit of additional NMR data. The gHMBC data also indicated that the two remaining methyl groups were correlating to two different quaternary olefinic carbon atoms. One of these methyl groups clearly was an *O*-methyl on the basis of chemical shift considerations (δ_C 66.4; δ_H 3.93), whereas the heteroatom attached to the second methyl group was not immediately obvious on the basis of chemical shift.

A first set pausible proposed structures for **1** was assembled based on the well-precedented structural motif of a β-carboline. Thus, the ring in substructure **e** was closed to yield a pyridoindole system to which was appended in position 1 (β-carboline numbering) a five-membered aromatic heterocycle in either of three orientations. This operation yielded structures **I**, **II**, and **III** in Figure 4.

With the heterocycle being a thiophene (X = S), the chemical shifts of the olefinic carbon atoms within the ring (δ_C 121.0, 137.0, 140.2, 144.6) appeared to be reasonable. However, the chemical shifts of the methyl group resonating upfield (δ_C 42.2; δ_H 3.13) were not explicable if both Z and

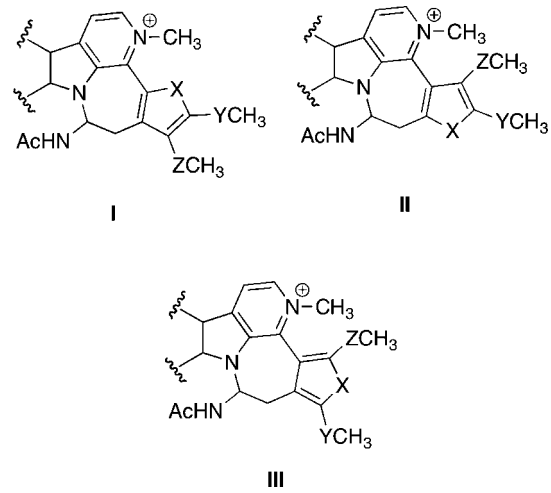

Figure 4. Candidate structures based on the β-carboline skeleton.

Y were oxygen.[6,7] If the heterocycle was made to be a furan (X = O), the chemical shifts of the β-carbon atoms within the ring were not reasonable. Moreover, the chemical shift of the putative thiomethyl group (Y or Z = S) was not in accord with that of thiomethyl furans in the literature.[8] Last, it appeared doubtful that the conjugation of the furan or the thiophene with the β-carboline would be sufficient to explain the UV−vis spectrum and hence the color of **1**. Closer chemical shift comparisons with β-carbolines in the literature revealed another problem: the chemical shift of the vinyl carbon atom next to the quaternary nitrogen atom, which was too far downfield in **1** by approximately 20 ppm in comparison to quaternized β-carbolines.[9,10] It thus appeared unlikely that compound **1** was indeed a pyridoindole derivative as in **I**, **II**, or **III**.

At this juncture, we returned to the chemical shift of the methyl group resonating at δ_C 42.2 and δ_H 3.13. Given the heteroatoms that remained to be assigned after substructure **g** and the aforementioned *O*-methyl group had been assembled, namely one sulfur and one oxygen atom, these chemical shifts were most readily explicable if the methyl were part of a methyl sulfoxide. The presence of such a group might also explain the doubling of the resonances seen in both the ^{1}H and the ^{13}C spectra of **1**.

With two heteroatoms now spoken for by the sulfoxide, all of the heteroatoms of the molecular formula had been accounted for. As three rings still had to be formed to account for all of the double-bond equivalents suggested by the molecular formula, the conclusion was inevitable that the putative five-membered ring had to be carbocyclic rather than heterocyclic.

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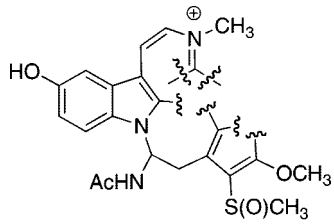

IV

Figure 5. Preassembly structure of **1** containing all atoms.

One of the limited number of variants by which the connections in structure **IV** (Figure 5) could be made without adding additional atoms results in an azafulvene. The presence of that moiety explains the blue color, which is otherwise not readily rationalized on the basis of the number of double bonds present. We made a similar argument recently in the structure elucidation of a new member of the prodiginine class of compounds.[11] For a listing of some known azafulvenes and their color see Table S-2 in the Supporting Information.

The placement of the *O*-methyl and the sulfoxide follows from NOE experiments: the methyl resonating at δ_H 3.93 shows NOEs to both the quaternary *N*-methyl as well as the sulfoxide methyl. It should therefore be placed in between them. This placement is corroborated by the aforementioned weak NOE between the sulfoxide methyl and the H_2-16ab protons.

Pseudocerosine is accompanied by a minor congener, the sulfone, as suggested by ESI-TOFMS (m/z [M^+] = 440.1) and by the ^{1}H NMR spectrum in which the previously noted doubling of signals is no longer evident and in which the sulfoxide methyl group is shifted downfield to δ_C = 44.8/ δ_H = 3.46 (for complete chemical shift information see Table S-1 in the Supporting Information).

In conclusion, we have established the structure of a new marine azafulvene pigment. Compound **1** is not appreciably cytotoxic at 25 μg/mL when tested against SKOV-3, a human adenocarcinoma cell line. While other indolic azafulvenes, the iheyamines, from the ascidian *Polycitorella* sp. are known, [12] the structure of **1** to the best of our knowledge is unique.

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Supporting Information Available: NMR spectra, spectral data, and structures of related materials. This material is available free of charge via the Internet at http://pubs.acs.org.

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